Remarks

By the present amendment, claims 4, 9-10, 15-16, 48 and 49 are revised to address issues raised in the Final Office Action. No new matter is entered by these amendments.

Applicant respectfully urges entry of these amendments after final response because the revisions do not necessitate a further search, and they place the claims in condition for allowance or, at least, in better condition for appeal.

The Final Office Action dated July 12, 2006, has been carefully considered. The amended claims and the following comments are believed to represent a complete response and to place the application in condition for allowance. Reconsideration is respectfully requested, therefore.

§112, Second Paragraph, Rejections

Claim 4 was rejected for a clerical error in referencing step "c" instead of step "d". Applicant has amended claim 4 to reference step "d" as suggested in the Action. Entry of this amendment and withdrawal of this rejection therefore is respectfully requested.

Claims 48 and 49 were rejected for allegedly being confusing, in view of the previous amendments to claim 1, from which they depend. Applicant appreciates the Examiner's careful attention to the claim language, and agrees that claims 48 and 49 no longer properly depend from claim 1.

Accordingly, Applicant has revised claim 48 into independent format, using language that is largely based on the version of claim 1 that was pending before the amendments of April 21, 2006. Applicant also has made claim 49 depend from claim 48 and include the recitations of claims 13 and 16, which were incorporated previously by virtue of the claim's dependence from claim 16 (which itself depends from claim 13, which in turn

depends from claim 1). Applicant believes that the amended claims clearly recite the embodiments at issue and, hence, that the indefiniteness rejection should be withdrawn.

With respect to the request entry of these amendments after final, Applicant notes that claims 48 and 49 recite embodiments largely identical to those already considered by the Examiner, for example, in the context of original claims 48 and 49, presented with the Amendment filed October 25, 2005. Indeed, on pages 4 and 5 the Action acknowledges that claims 48 and 49, as presented prior to the claim 1 revisions of April 21, 2006, were "supported by the specification."

35 USC §112, First Paragraph – Written Description

Claims 1, 4-10, 12-16, 18-19 and 48-50 stand rejected for alleged lack of written description with respect to the term "non-human host cell." Applicant respectfully traverses this rejection.

Page 4 of the Action acknowledges that the specification teaches the use of non-human host cells, such as bacterial cells, insect cells, yeast cells, and plant cells, as host cells. Nevertheless, the Action cites MPEP § 2173.05(i) for the proposition that "[a]ny negative limitation . . . must have basis in the original disclosure," apparently reading this provision as imposing a requirement for support in haec verba for a negative proviso. Yet the MPEP imposes no such requirement, which would contravene settled law on the written description requirement.

As noted in MPEP § 2173.05(i), "there is nothing inherently ambiguous or uncertain about a negative limitation." The MPEP does state that "[a]ny negative limitation . . . must have **basis** in the original disclosure," but this does not require support in haec verba. To the contrary, the MPEP explains that "a lack of literal basis in the specification for a negative limitation may not be sufficient to establish a prima facie case for lack of descriptive support," and cites MPEP § 2163 for further information.

The latter provisions plainly state that "the subject matter of the claim need not be described literally (i.e., using the same terms or in haec verba) in order for the disclosure to satisfy the description requirement." Instead, it explains, the "fundamental factual inquiry" for written description "is whether the specification conveys with reasonable clarity to those skilled in the art that, as of the filing date sought, applicant was in possession of the invention as now claimed."

MPEP § 2163.04 sheds further light on this issue, requiring that a rejection for lack of written description include "reasons why a person skilled in the art at the time the application was filed would not have recognized that the inventor was in possession of the invention as claimed in view of the disclosure of the application as filed." The rejection here fails to include such reasoning.

The specification provides ample support for the recitation of a "non-human host cell," and clearly conveys to the skilled artisan that Applicant had possession of the claimed invention at the time of filing. At noted at page 4 of the Action, the specification (page 7, lines 3-4) teaches that bacterial cells, insect cells, yeast cells, plant cells and mammalian cells are suitable as host cells. The specification additionally teaches that the protein "may also be produced in an edible food source, such as animal milk, or in an edible crop." Specification, page 13, lines 27-29. Applicant respectfully submits that these teachings, particularly when read in view of the disclosure as a whole, plainly convey to the skilled artisan Applicant's possession of the invention with regard to non-human host cells. Indeed, because aspects of the invention relate to "methods for recovering recombinantly produced polypeptides," (see, e.g., Specification, pg. 1, lines 3-4, & Abstract), those skilled in the art surely would understand that references to "mammalian" host cells includes "non-human" host cells, particularly in view of the reference to production in "animal milk."

Only an imposition of an in haec verba requirement, in contravention of the PTO's own rules, could substantiate a written description rejection keyed to the present recitation of

"non-human" host cells. Precisely because such a requirement is contrary to law, the present rejection of claims 1, 4-10, 12-16, 18-19 and 48-50 is improper and should be withdrawn.

35 USC §112, First Paragraph – Enablement

Claims 1, 4-10, 12-16, 18-19 and 48-51 were rejected for alleged lack of enablement. Applicant respectfully traverses this rejection.

While acknowledging that the claimed invention is enabled with respect to recombinant production in an isolated host cell or in a bacteria, yeast or insect cell, the Action continues to question enablement with respect to recombinant production in "any non-human host," or in "any plant." Applicant respectfully maintains its position that the full scope of the pending claims is enabled.

The Action continues to focus improperly on the enablement of "gene transfer." The present invention relates to the discovery that a recombinant polypeptide of interest can be obtained by methods comprising recombinantly producing a fusion protein comprising a full-length chymosin pro-peptide and the polypeptide of interest, and contacting the fusion protein with a mature form of an autocatalytically maturing aspartic protease that is capable of cleaving the chymosin pro-peptide from the fusion protein to release the recombinant polypeptide. While the claimed methodology involves the recombinant production of proteins in a host cell, recombinant protein production per se is not the focus of the invention. Indeed, the evidence of record demonstrates that the state of the recombinant protein production art was advanced at the time the application was filed.

As set forth in MPEP § 2164.03, the "more that is known in the prior art about the nature of the invention . . . the less information needs to be explicitly stated in the specification." Indeed, MPEP § 2164.05(b) provides that "[t]he specification need not

disclose what is well-known to those skilled in the art and preferably omits that which is well-known to those skilled and already available to the public." In keeping with these guidelines, the instant specification was not required to provide detailed teachings and examples of recombinant protein production in various host cells, because that information was known in the art and readily available to the skilled artisan at the time of filing, as demonstrated by the numerous representative references submitted with Applicant's previous response.

That the Examiner was able to uncover three articles that discuss ongoing issues in the field of recombinant protein production does not show that it would have required an undue amount of experimentation for someone skilled in the art to practice the invention. Instead, these articles support Applicant's position as to the advanced state of the art, because they address issues that have arisen as recombinant protein production has moved out of the research laboratory into the commercial world.

The Action cites Dyck for stating that "the generation of transgenic domestic animals is <u>difficult</u> . . . " and that "current methods . . . are relatively <u>inefficient</u> and <u>time-consuming</u>" (emphasis added). These alleged problems are purely commercial considerations that have no bearing on the enablement of the claimed methods. Indeed, the entire focus of Dyck is the evaluation of various transgenic systems as "bioreactors" for large-scale production of proteins, and Dyck starts with the premise that "[t]he ability of transgenic animals to produce complex, biologically active recombinant proteins . . . has stimulated a great deal of interest in this area."

Dyck itself cites numerous categories of successful transgenic protein production, including transgenic milk ("Foreign proteins are commonly reported to be expressed in transgenic milk at rates of several grams per litre," pg. 395); the blood of transgenic pigs ("[P]igs producing human haemoglobin in their own circulatory system have been produced," pg. 395); methods using retroviruses ("[R]etroviruses have been used to successfully produce transgenic mice and viral integration of recombinant sequences

into bovine embryos to produce transgenic calves have been reported," pg. 396); methods using embryonic stem (ES) cells or primordial germ (PG) cells ("Reviews of the literature indicate that the production of chimeric animals with ES or PG cell technology has been applied successfully in mice, rabbits, pigs, cattle and poultry," pg. 397), and methods using pronuclear microinjection, which Dyck characterizes as being "the most straightforward and consistently successful means of gene transfer for most species" (pg. 397). This is hardly evidence of non-enablement.

The Action cites Vain for stating that "transgene expression in plants remains largely unpredictable," but that statement is taken out of context. Reading the complete sentence (at pg. 878, col. 2) reveals that the "unpredictable" factors being noted are "variation in expression levels and stability between independently transformed plants," not the ability to achieve transgenic expression per se. In Vain's own experiments, 100% of 95 independently transformed rice plants successfully expressed one of two transgenes, and 87% expressed both transgenes. Thus, Vain does not support an assertion that undue experimentation is required to produce transgenic proteins in plants. Instead, Vain's focus is on transgene behavior in the progeny of transformed plants, an issue that is wholly irrelevant to the enablement of the instant claims.

The Action cites Potrykus for the premise that gene transfer in cereals is largely unsuccessful, but the Action's reliance on this article to support an enablement rejections is misplaced. Potrykus presents an admittedly "subjective" review of different methods that have been used to effect gene transfer in cereal crops "and their potential agronomic utility" (emphasis added). Thus, this article, like those discussed above, relates to issues encountered on the road to commercialization. While Potrykus criticizes a number of different transgenic methods, it also acknowledges methods that have proven successful, including methods using agrobacterium or agroinfection to transform dicots and methods using protoplasts for direct gene transfer of cereals. In fact, the "Note added in proof" at page 542 reports Potrykus' own work to establish "what we believe is proof of the recovery of transgenic offspring of Indica-type rice."

That work, reported in Datta et al., Bio/Technology (1990) 736-40 (copy attached), is said to result in "a simple and reproducible method of transformation of an important food crop." Thus, Potrykus does not undermine the enablement of the present invention with respect to recombinant protein production in plants.

Applicant also questions the validity of Potrykus as an accurate reflection of the state of the art. As noted above, Potrykus itself states that it presents a "subjective" discussion. Moreover, Potrykus admits that it includes "several statements. . . for which no solid experimental data are available." The evidentiary value of Potrykus' critiques therefore pales in view of the numerous references of record that present scientific data demonstrating the successful production of transgenic proteins in plants, including Potrykus' own work.

In citing Dyck, Vain and Potrykus against Applicant, the Action loses sight of the fact that § 112 does not require an applicant "to enable one of ordinary skill in the art to make and use a perfected, commercially viable embodiment." MPEP 2164. All that § 112 requires is that the specification enable those skilled in the art to practice the claimed invention without an undue amount of experimentation. Because Applicant has demonstrated that recombinant protein production in a wide variety of hosts (including animals, bacteria, insects and plants) was well-developed at the time the present application was filed, the instant enablement rejection is improper, and should be withdrawn.

35 USC §103

The Action maintains the following obviousness rejections:

the rejection of claims 1, 4, 6-9, 13, 15, 19 and 51 over Ward in view of Walsh and Yonezawa;

the rejection of claim 5 over Ward, Walsh and Yonezawa, and further in view of Fine, as set forth in the previous Action; and

the rejection of claims 14 and 50 over Ward, Walsh, and Yonezawa, and further in view of Dunn.

Applicant respectfully traverses these rejections for the reasons set forth in Applicant's previous responses, as supported by the Declaration of Dr. Moloney. As stated previously, there is simply no teaching or motivation in the prior art of preparing a recombinant polypeptide of interest by producing a fusion protein comprising a chymosin pro-peptide and the polypeptide of interest, and using a mature aspartic protease to cleave the chymosin pro-peptide sequence from the fusion protein to release the recombinant polypeptide of interest, as claimed. As demonstrated previously, those skilled in the art had no reasonable basis for expecting that an aspartic protease would be capable of cleaving a chymosin pro-peptide from a fusion protein to release the recombinant polypeptide, and did not know, for example, whether the aspartic protease would cleave the recombinant polypeptide at undesired sites and/or would cleave off too many or too few amino acid residues around the junction between the pro-peptide and the recombinant polypeptide. Without an assurance of accurate cleavage, there was no motivation to have employed an aspartic protease as presently claimed.

Because it is only the instant specification that recognizes and teaches that aspartic proteases are capable of cleaving a chymosin pro-peptide from a fusion protein to release a recombinant polypeptide of interest, these obviousness rejections are improperly founded on hindsight, and should be withdrawn.

The Action newly rejects claims 10 and 16 as obvious in view of Ward, Walsh and Yonezawa, and further in view of Huber (U.S. Patent No. 4,180,559) and Fan (U.S. Patent No. 4,774,183). The Action explains that this rejection is based on an interpretation of claims 10 and 16 that does not require step d) of the recited method to be effected in vivo, but that reads those claims as encompassing methods effected in vitro, but under "in vivo conditions." Applicant believes that the foregoing amendments

to claims 10 and 16 obviate this rejection. As amended, claims 10 and 16 recite that "step d) is effected in vivo." Because the cited references do not teach or suggest such a method, the obviousness rejection should be withdrawn.

CONCLUSION

In view of the foregoing, we submit that the application is in order for allowance and an early indication to that effect would be greatly appreciated. Should the Examiner like to discuss the matter, he is kindly requested to contact Micheline Gravelle at 416-957-1682, or Courtenay Brinckerhoff at 202-295-4094, at his convenience.

The Commissioner is hereby authorized to charge any deficiency in fees (including any claim fees) or credit any overpayment to our Deposit Account No. 02-2095.

Respectfully submitted,

By Courtenay C Brinckerhoff
Micheline Gravelle
Reg. No. 40,261 Reg No. 37,288

Bereskin & Parr
Box 401, 40 King Street West
Toronto, Ontario
Canada M5H 3Y2
Tel: 416-957-1682
Fax: 416-361-1398

RESEARCH

GENETICALLY ENGINEERED FERTILE INDICA-RICE RECOVERED FROM PROTOPLASTS

Swapan K. Datta*, Alex Peterhans[1], Karabi Datta and Ingo Potrykus

Swiss Federal Institute for Technology, Plant Sciences, ETH-Zentrum, CH-8092, Zurich, Switzerland. [1]Present address: Rockefeller University, 1230 York Ave., NYC, NY 10021. *Corresponding author.

We have established an efficient protocol for plant regeneration from haploid Indica-type rice protoplasts. Incubation of these protoplasts with the selectable hygromycin phosphotransferase *(hph)* gene expressed under control of the 35S promoter of cauliflower mosaic virus (CaMV) and polyethyleneglycol (PEG), and subsequent culture in the presence of hygromycin B, led to the recovery of numerous resistant clones from which 77 plants were regenerated. Data from Southern analysis and enzyme assays proved that the transgene was stably integrated into the host genome and expressed, and that it was inherited in offspring.

Rice *(Oryza sativa)* is the world's most important crop plant[1]. Indica-type rice varieties feed more than 2 billion people, predominantly in developing countries[2]. Despite a variety of approaches, transgenic cereals can be produced so far only by methods of direct gene transfer[3,4], which require competent protoplasts[5]. Dividing cereal protoplasts, from which plants can be regenerated, are isolated from embryogenic cell cultures[6]. Such cultures are normally derived from immature embryos. We have established an embryogenic suspension culture from immature pollen grains[7] of Indica type rice *Oryza sativa* var. Chinsurah Boro II. Plants can subsequently be regenerated from protoplasts of these cultures[8]. We report here the transformation of Indica-type rice and transmission of the transgene to the progeny.

RESULTS

Microspore-derived embryogenic cell suspension culture. An embryogenic suspension culture was established via anther float culture from immature microspores of Indica-type rice as described recently[8] and maintained under diffuse light for isolation of protoplasts (Fig. 1a). The culture was composed of clusters of small, cytoplasm-rich cells and required subculturing every 5 days (Fig. 1b).

Protoplast culture, transformation and regeneration. Protoplasts were isolated from 5–12 month old cultures by incubation in an enzyme mixture that yielded an average 4×10^6 protoplasts/g cell suspension culture (Fig. 1c). No undigested clumps of cells were detected in the protoplast preparations. However, a few spontaneously fused protoplasts were observed. Nurse cultures or feeder layers were not required for culturing of isolated protoplasts, divisions and regeneration of plants (Fig. 1 d–h). PEG-mediated transformation was performed as described in the Experimental Protocol.

Treatment of protoplasts with DNA and PEG 6000 for a short time (10 min) was important in obtaining high frequencies of protoplast survival, divisions and subsequent plant regeneration. To allow selection for transformed cell clones, plasmid pGL2, which carries the *hph* gene under control of the 35S promoter and polyadenylation signal of CaMV, was used (Fig. 2a). The *hph* gene confers hygromycin resistance to transformed cells. Hygromycin resistant (Hm^r) cell clones were transferred to semi-solid N6 medium[9] containing 25 μg/ml hygromycin B, 1 mg/l 2,4-D and 0.3% agarose to allow for continued proliferation. No clones developed in the control samples (Fig. 1e, left dish). After 2–4 weeks, compact embryogenic clones were transferred to modified MS culture medium[10] without hygromycin B and somatic embryos developed (Fig. 1f). All cultures to this point were kept in the dark. Transfer of somatic embryos to hormone-free, modified MS medium under light led to the outgrowth of multiple shoots and roots (Fig. 1g). Plants regenerated from hygromycin-resistant clones grew to maturity in the greenhouse and set seeds (Fig 1h). These plants resembled control plants regenerated from untreated protoplasts or grown from seeds. Of the 77 plantlets recovered 24 were grown to maturity (Table 1).

Molecular data: Southern analysis. Total genomic DNA was isolated from 10 hygromycin-resistant primary regenerants and analysed by the method of Southern[11]. In all cases, hybridization with a radioactively-labeled probe specific for the protein coding region of *hph* demonstrated that this gene had integrated into the genome (data not shown). Figure 2b shows the Southern blot data for one representative primary transgenic plant (lanes 3–

TABLE 1 Recovery of transgenic Indica-rice plants from protoplasts treated with plasmid pGL2 and PEG.

Expt. No.	Antibiotic	Con. (μg/ml)	Plasmid	No. of Protoplasts Used ($\times 10^6$)	No. of Hm^r Clones (28d)	No. of Colonies Tested for Regeneration	Plants Regenerated (Green)	Plants Regenerated (Albino)
1	Hm	25	pGL2	6.5	200	60	—	—
2	Hm	25	pGL2	8.0	350	280	73	16
3	Hm	25	pGL2	8.5	116	22	4	—
4	Hm	25	pGL2	5.8	28	nt	nt	nt
C_1	—	—	—	8.0	2260*	430	25	2
C_2	Hm	25	—	8.0	—	—	—	—

*No. of colonies without selection; Hm=hygromycin; —indicates no response; nt=not tested; C_1 control without DNA and selection; C_2 control without DNA but with selection

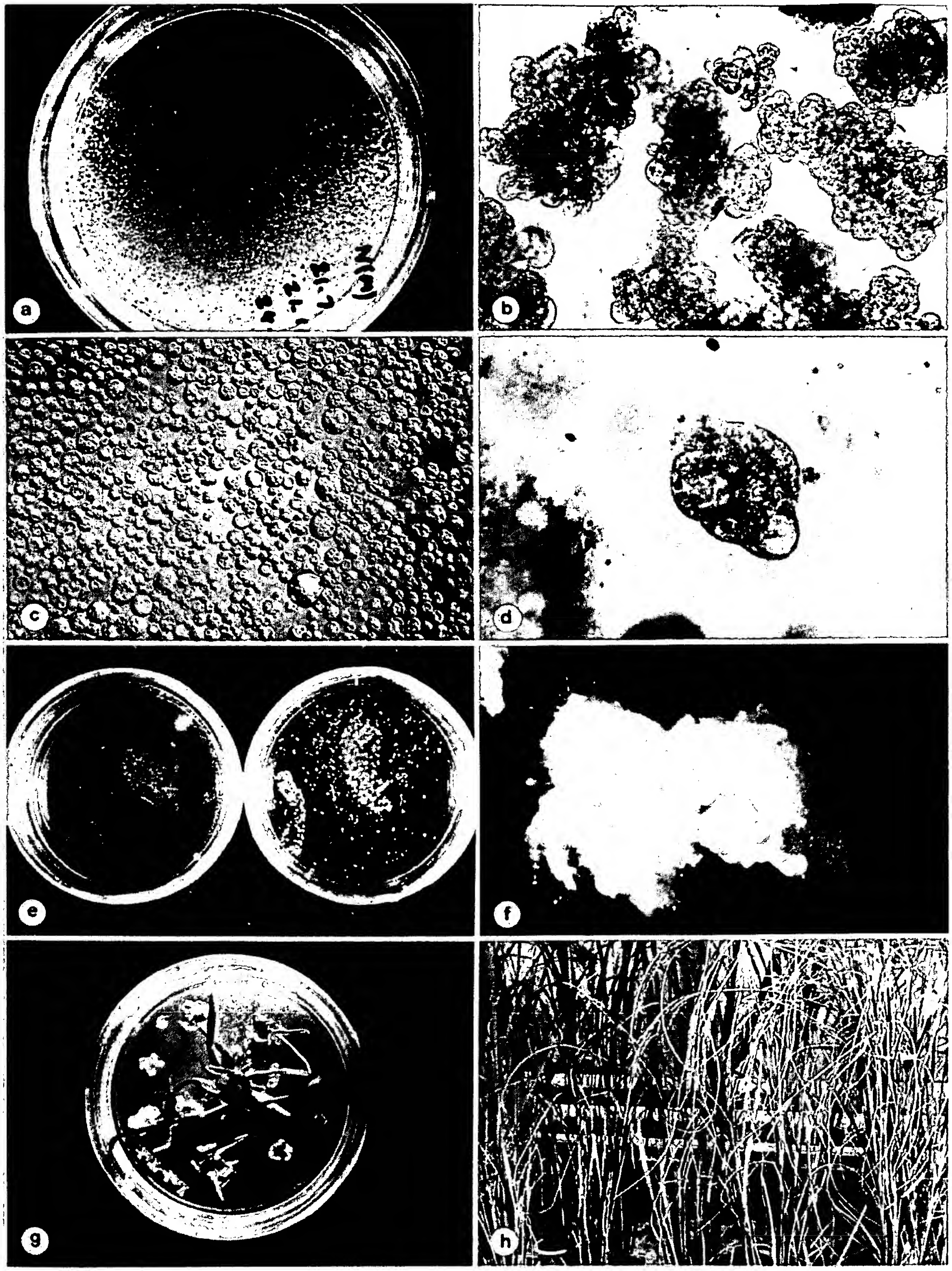

FIGURE 1 Regeneration of transgenic fertile indica-rice plants from protoplasts. (a) Embryogenic cell suspension (ECS) culture of microspore origin; (b) Enlarged view of ECS showing compact cytoplasmic-rich cell groups; (c) Protoplasts isolated from 5 month old ECS; (d) A protoplast derived group of cytoplasm-rich cells; (e) Hygromycin-resistant clones proliferating only from protoplast populations treated with plasmid pGL2 and PEG (right dish) and not in control treatment (left dish); (f) Resistant clones developing somatic embryos; (g) Differentiation of multiple shoots with roots in the light; (h) One primary transgenic plant with seeds and several offspring thereof.

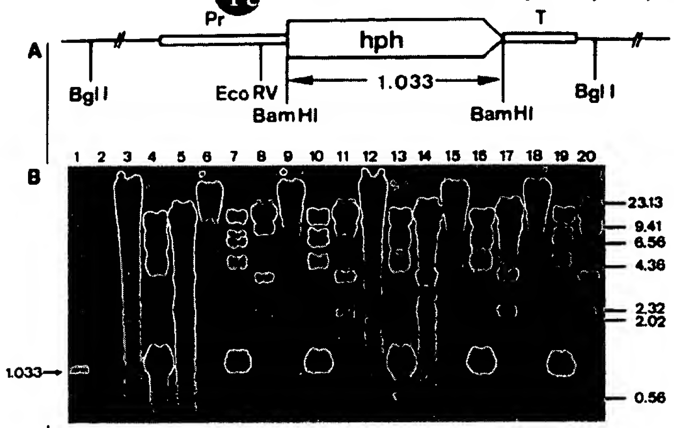

FIGURE 2 Molecular analysis of transgenic plants and offspring. (a) Part of plasmid pGL2 containing the bacterial gene for hygromycinphosphotransferase *(hph)* under the control of the expression signals of the 35S transcript of CaMV. For the transformation experiments plasmid pGL2 was cut with BglI releasing the fragment shown. The fragment contains a single EcoRV site within the promoter region; (b) Southern data are presented for one representative primary transgenic plant (lanes 3–5), and five offspring of this plant (lanes 6–8, 9–11, 12–14, 15–17, 18–20). Lane 1 represents a 3 copy reconstruction of the transforming plasmid cut with BamHI, lane 2 contains DNA from control (untransformed) rice plant, also cut with BamHI, samples are arranged in groups of three each. The first lane of these triplets (3, 6, 9, 12, 15, 18) contains undigested DNA, the second lane (4, 7, 10, 13, 16, 19) contains DNA restricted with BamHI (releasing a 1.033 kb fragment characteristic of the protein-coding region of the gene), the third lane (5, 8, 11, 14, 17, 20) contains DNA restricted with EcoRV (yielding border fragments between transforming DNA and host DNA). The size markers in kilo base pairs are derived from lambda DNA digested with HindIII.

5), and 5 independent offspring thereof (lanes 6–20). The integration pattern of the transgene in the offspring is identical to that of the parental plant. In all plants, hybridization of undigested DNA with the *hph*-specific probe showed that the transgene had integrated into the genome (Fig. 2b, lanes 3, 6, 9, 12, 15, 18). After restriction digestion with BamHI the expected 1.033 kb fragment characteristic of the coding sequence of *hph* was observed (Fig. 2b, lanes 4, 7, 10, 13, 16, 19; compare Fig. 2a). Digestion with EcoRV, which cuts once within the plasmid pGL2, produced junction fragments within the transforming DNA or with the host genome (Fig. 2b, lanes 5, 8, 11, 14, 17, 20). There was no hybridization to control material (Fig. 2b, lane 2).

Enzyme assay. Evidence demonstrating the presence of the transforming DNA and resistance to hygromycin B were complemented by enzymatic proof that the gene is functional; specific phosphorylation of the antibiotic was observed using protein extracts prepared from transgenic plants (Fig. 3). The plants taken for the enzyme assay were the same as those used for the Southern analysis shown in Figure 2b. The assay for hygromycin phosphotransferase activity was positive for the primary transgenic regenerant (Fig. 3, lane 1) and the five offspring plants analyzed (Fig. 3, lanes 3, 5, 7, 9, 11). The enzymatic evidence was also confirmed by growth of (selfed) seedlings derived from transgenic plants on 40 μg/ml hygromycin. They all developed into healthy plants under these conditions. So far, 31 seeds derived from 5 primary transgenic plants tested, were all hygromycin resistant, whereas 42 seeds from protoplast derived control plants were sensitive. Control plants germinated poorly, developed brown roots, ceased growth and died.

DISCUSSION

Transformation of rice, by electroporation[12,14,15] and by PEG[13] has been so far reported only with japonica types[12–15]. Electroporation has also been used for transformation in maize[16,17]. In these publications, authors report the absolute requirement of either nurse culture[15] or feeder layer cultures[16–18] for proliferation of rice and maize protoplasts. However, it is also possible that rice plants (both japonica and indica) could be obtained without any nurse or feeder cultures[8,13,19]. Moreover, nurse culture reduces the growth of the transformed colonies[14]. A heat-shock treatment (5 minutes at 45°C) to the recipient protoplasts prior to addition of the plasmid followed by PEG as reported to be beneficial for rice transformation[13,14] did not improve the transformation frequencies in our experiments with Indica-rice (data not shown). Perhaps, microspore-derived embryogenic cell suspension are, to our experience, well suited for reproducible production of transgenic colonies (Table 1). Transgenic plants obtained from protoplasts-derived clones via somatic embryogenesis resembled seed-derived plants.

Southern data are presented for one representative primary transgenic rice plant and five offspring of this plant. The data demonstrate (a) integration of the transforming plasmid DNA into high molecular weight DNA; (b) presence of the expected 1.033 kb BamHI fragment in the primary transgenic plant and offspring; and (c) hybrid fragments between rice DNA and transforming DNA. From the comparison of the intensity of the 1.033 kb BamHI fragment of the transgenic plants with the corresponding fragment of the control plasmid in a reconstitution experiment we estimate that approximately 50–100 copies of the plasmid are present in the transformants. Southern data obtained from five independent offspring plants derived from the same primary regenerant demonstrated identical integration pattern of the transforming DNA in all plants (Fig. 2b). Southern blot analysis of five additional offspring plants derived from the same primary transformant revealed exactly the same results (data not shown). All 10 progeny plants derived from the same primary regenerant were Hm^r. Two hypotheses could explain these data: either on the basis of a hemizygous primary transformant having integrated the transforming DNA into two or more independent genomic loci as described for tobacco[20]. In this case, a segregation of the hybridizing bands resolved on the Southern blots should be expected in the offspring plants, which was not observed (Fig. 2b). Alternatively, since the starting material for protoplast isolation and transformation was a microspore derived cell suspension and thus eventually being haploid at the time used for transformation, the recovered fertile primary transgenic plants could be homozygous. The lack of segregation of the integration pattern of the foreign DNA in the offspring indicates that the primary transformant is indeed homozygous. Therefore, information concerning the number of independent genomic integration sites can not be obtained from the analysis of first self-pollinated R_1 progeny. Further genetic analyses of more transgenic plants of independent experiments and determination of the ploidy levels of the cell suspension before transformation should clarify this point.

Resistance to hygromycin B is based on inactivation of the antibiotic via the transfer of the γ-phosphate from ATP[21]. The enzyme activity was absent in control rice tissue and present in both transformed leaves of the primary regenerant described here and in progeny plants thereof (Fig. 3). These data show that these plants not only carry the transgene but also express it.

We report here a simple and reproducible method of

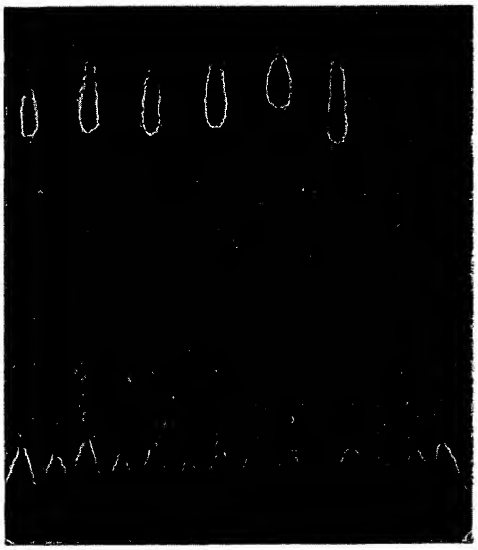

FIGURE 3 Enzyme assay for hygromycinphosphotransferase of primary transgenic and offspring plants. The plants assayed are identical to those used in the Southern blot analysis shown in Figure 2b. The autoradiogram of the TLC plate shows labelled ATP at the start in all lanes and labelled hygromycin B in lanes 1, 3, 5, 7, 9, 11. Even numbers represent enzyme reactions carried out without Hygromycin B, odd numbers represent reactions containing Hygromycin B. Lanes 1, 2 contain crude protein extract derived from the primary transgenic plant; lanes 3–12 contain extracts derived from five offspring of the same primary plant; lanes 13 and 14 contain extracts isolated from an untransformed control rice plant.

transformation of an important food crop, Indica-type rice and transmission of the foreign gene to the progeny. As co-transformation has been established as an efficient and routine procedure for the recovery of plants transgenic for non-selectable genes[15,22] and transgenic plants resistance to insect pests have already been tested and grown in the field[23], it should now be feasible to approach the production of Indica-type rice plants transgenic for agronomically more interesting genes.

EXPERIMENTAL PROTOCOL

Plant material. Experiments were carried out with one Indica-type rice variety (Chinsurah Boro II, IRRI Acc. No. 11484, kindly supplied by the International Rice Research Institute, Manila, Philippines and Rice Research Station at Chinsurah, West Bengal, India). Growth of donor plants, culture of microspores, embryogenic cell suspensions and protoplasts have been described earlier[7,8].

Plasmid construct. Plasmid pGL2 was obtained by cloning the *hph* gene as a BamHI fragment derived from plasmid pGL88[21] (under control of 35S promoter and polyadenylation signal of CaMV[24]) into the BamHI site of plasmid pDH51[25]. The transforming DNA used for direct gene transfer was cut with BglI releasing the fragment shown in Figure 2a. Plasmid pGL2 contains a single EcoRV site in the promoter region of the gene. Carrier DNA was prepared by dissolving calf thymus DNA (Sigma) sheared to an average size of approximately 4 kb in water and filter sterilized.

Protoplast culture, transformation and production of transgenic plants. An embryogenic cell suspension was obtained from dividing microspores and maintained for more than a year under diffuse light (7 μE s^{-1} m^{-2} at 80 rpm) for 24 h photoperiod in a medium described earlier[8]. Protoplasts were isolated from 5-20 month old cultures by incubation in the following enzyme mixture: 4% cellulase onozuka RS (Kinki Yakult, Japan), 1% macerozyme R10 (Kinki Yakult, Japan), 0.02% pectolyase Y-23 (Seshin Pharmaceutical, Japan) (all w/v) dissolved in 0.4 M mannitol, 6.8 mM $CaCl_2$, pH 5.6 adjusted before filter sterilization. Protoplasts were washed three times by sedimentation in 0.4 M mannitol, 6.8 mM $CaCl_2$ and resuspended in a final density of 1.5×10^6/ml. Aliquots of 0.4 ml of the protoplast suspension were mixed with BglI-digested pGL2 plasmid DNA (6 μg) and calf thymus, carrier DNA (28 μg). Immediately after mixing the protoplasts with DNA, 0.4 ml of PEG solution (polyethyleneglycol MW 6000, Merck, 40% w/v) was added dropwise and the mixture of protoplasts, DNA and PEG incubated at 20°C for 10 min[26]. Protoplasts were then cultured with 8 ml of washing solution (0.4M mannitol, 0.1% MES, pH 5.6) slowly and centrifuged to remove the PEG. Subsequently, aliquots of 0.5 ml protoplasts suspension were mixed gently (in a 35 mm Falcon dish) with 0.5 ml of modified N6 medium containing 2.4% (w/v) agarose (Sea Plaque, FMC), 1 mg/l 2,4-D, 0.4 M sucrose, 28 mM glucose and allowed to gel. Following incubation in the dark at 27°C for 7 days, the agarose gel was cut into segments, which were transferred to 5 ml N6 medium with the following modifications: 1 mg/l, 2,4-D, 175 mM sucrose, 28 mM glucose and 0.3% (w/v) agarose and continued in 6 cm Sterilin plastic plates as bead type culture[27]. Hygromycin B was added to the medium at day 14 after protoplast isolation to a final concentration of 25 μg/ml. After 4–5 weeks of selection, visible colonies were transferred onto soft agarose N6 medium containing 1 mg/l 2,4-D, 175 mM sucrose, 28 mM glucose, 0.3% agarose, 25 μg/ml hygromycin B. Following 2–4 weeks at 24°C in the dark, cell colonies of ca. 1.5 mm diameter and developing somatic embryos were transferred to modified MS[10] culture medium containing 146 mM sucrose, 2 mg/l kinetin, 1 mg/l NAA, 0.8% agarose, 300 mg/l casein hydrolysate (ICN). Incubation in the light (24 μE s^{-1} m^{-2}), 16 h photoperiod led to the development of plantlets with multiple tillers with roots from somatic embryos. These were transferred to MS agar medium without hormones and containing 58 mM sucrose for 2–3 weeks. At this stage the agar was removed from the roots and the plantlets were transferred to potting compost and adjusted to greenhouse conditions. In the greenhouse, the plants grew to maturity and set seeds.

Progeny test for resistance to hygromycin. Seeds derived from self pollinated plants were surface sterilized in 1.8% (v/v) sodium hypochlorite and washed extensively with sterile distilled water. MS culture medium without hormones and vitamins either liquid or solidified with 0.8% agar, 1.0% sucrose, containing 40 μg/ml hygromycin B was used for germination of seeds. Seeds were incubated under light (24 μE s^{-1} m^{-2}), 16 h photoperiod at 24°C for 14 days.

Molecular analysis of transgenic plants. Total genomic DNA was isolated from leaf tissue of hygromycin-resistant and control plants. The leaves were freeze dried and ground in a mortar and pestle until a powder was obtained. DNA was extracted using the CTAB method[28]. Three μg of genomic DNAs was digested with BamHI or EcoRV restriction enzymes. Following electrophoresis through 0.8% agarose, DNA was transferred to Hybond-N nylon membranes. Hybridizations were done according to the instruction of the manufacturer (Amersham). The radioactive probe was prepared by the random primer method using (α-^{32}P)dATP[29]. The probe consisted of the protein-coding region of the *hph* gene (1.033 kbp BamHI fragment isolated from plasmid pGL2).

Assay for hygromycin phosphotransferase. The enzyme assay was carried out as previously described[30], modified according to personal communication by R. D. Shillito (CIBA Geigy, Research Triangle Park, North Carolina, USA). Leaves were frozen in liquid nitrogen and ground with pestle and mortar in extraction buffer (0.05 M Tris-HCl, pH 7.0, 10% glycerol, 0.1 mM phenylmethyl sulphonyl fluoride) (100–200 mg tissue/100 μl) in the presence of acid washed sea sand at 4°C. The samples were then centrifuged at 14000 rev/min for 5 min at 4°C and the supernatant used for the reaction. The enzyme reactions were carried out in 10 μl volumes containing 50 mM Tris-maleate, pH 7.0, 50 mM $CaCl_2$, 0.05 mM ATP, 0.4 μl (γ-^{32}P)ATP (10 mCi/ml; 3000 Ci/mmol), 62 μg hygromycin B and 5.6 μl crude extract. Reactions were carried out with and without hygromycin. Incubation was for 30 min at 37°C. One μl aliquots from the reaction mixtures were applied to a PEI-cellulose F TLC plate (Merck), which was developed in 50 mM sodium formate/formic acid, pH 5.4. The plates were dried prior to autoradiography.

Acknowledgments

We thank Paul Frick for his excellent care of the rice plants in the greenhouse and Laura Sarokin, J. Paszkowski and M. W. Saul for their helpful discussions and critical reading of the manuscript.

Received 15 April 1990; accepted 1 June 1990.

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